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Salmonella Enteritidis–Induced Alteration of Inflammatory CXCL Chemokine Messenger-RNA Expression and Histologic Changes in the Ceca of Infected Chicks

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SUMMARY. To understand better the events in early avian host immune responses to *Salmonella* Enteritidis (SE), we examined messenger-RNA (mRNA) expression for eight genes: CXCLi1[K60], CXCLi2 [IL-8/CAF], interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-12α, IL-12β, and gallinacin (Gal)-2 in the ceca of young chicks 1 wk postinoculation with SE. Cecum tissue sections were stained and evaluated for the presence of macrophages, lymphocytes, heterophils, and apoptotic cells following SE infection. With the use of quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), SE infection was associated with a significant ($P < 0.01$) upregulation of cecal CXCLi1 and CXCLi2 mRNA expression. Infection with SE was also associated ($P < 0.05$) with increased staining for macrophages and decreased apoptosis (single-stranded DNA [ssDNA]) in cecal tissue sections when these sections were compared with those of uninfected animals. Changes in chemokine expression and cell population dynamics are a direct result of SE infection, as uninfected animals do not show these alterations. Thus, these SE-induced changes reflect the host immune response to SE in young chickens.

RESUMEN. Alteración de la expresión del ARN mensajero de la quimioquina CXCL y cambios histopatológicos inducidos por *Salmonella* Enteritidis en el ciego de pollos infectados.

Con la finalidad de entender mejor los eventos en la respuesta inmune temprana de las aves a la *Salmonella* Enteritidis, una semana posterior a la inoculación de pollos jóvenes con *Salmonella* Enteritidis se examinó la expresión de RNA mensajero de ocho genes: CXCLi1 [K60], CXCLi2 [IL8/CAF], interferón (IFN)-γ, interleuquina (IL)-1β, IL6, IL12α, IL12β y Gallinacin (GAL)-2. Posterior a la infección con *Salmonella* Enteritidis, se tiñeron cortes de tejido del ciego y se evaluaron para la presencia de macrófagos, linfocitos, heterófilos y células apoptóticas. Mediante el uso de la prueba de reacción en cadena por la polimerasa-transcriptasa reversa, se estableció la asociación de la infección con *Salmonella* Enteritidis y un incremento significativo ($P < 0.01$) en la expresión del ARN mensajero de los genes CXCLi1 y CXCLi2. La infección con *Salmonella* Enteritidis también se asoció ($P < 0.05$) con el incremento en la tinción para macrófagos y la disminución de la apoptosis (ADN de cadena sencilla) en los cortes de ciego, cuando se compararon con los cortes provenientes de animales no infectados. Los cambios en la expresión de quimoquinas y la dinámica de las poblaciones celulares son un resultado directo de la infección con *Salmonella* Enteritidis, puesto que las aves no infectadas no muestran estas alteraciones. En consecuencia, estos cambios inducidos por la *Salmonella* Enteritidis reflejan la respuesta inmune en aves jóvenes a la bacteria.

Key words: bacterial infection, chemokines, cytokines, monocytes/macrophages

Abbreviations: cfu = colony-forming units; Ct = cycle threshold; DNases = deoxyribonucleases; Gal = gallinacin; H&E = hematoxylin and eosin; IFN = interferon; IgM = immunoglobulin M; IL = interleukin; LB broth = Luria-Bertani broth; mRNA = messenger RNA; pi = postinfection; RT-PCR = reverse transcriptase–polymerase chain reaction; SE = *Salmonella* Enteritidis; ssDNA = single-stranded DNA; TGF-β = transforming growth factor beta

Poultry eggs and meat contaminated with *Salmonella* Enteritidis (SE) continue to be very significant sources of human food poisoning in the United States (28,30,34), with approximately 35% of U.S. flocks testing positive for SE in 2000 (6). Antimicrobial resistance in SE strains is widespread, with more than 90% being resistant to at least one antimicrobial drug; over 50% of SE strains isolated from poultry have been found to be multidrug resistant (5). A more thorough understanding of the avian immune response to SE may provide novel insights for vaccine development as well as enhanced host immunity to this problematic bacterial pathogen, with an overall goal of reducing human illness.

Once ingested, SE rapidly travels to the cecum within about 12 hr, and later becomes a systemic infection, reaching the spleen and liver in 24–48 hr (39). Bacterial colonization of the spleen and liver is undetectable 4 wk postinoculation, indicating chickens are able to resolve infection in these organs; however SE will persist in the cecum for up to 16 wk (11,32). Additionally, half of infected hens were shedding SE in feces from 18 to 24 wk (11), suggesting that long-term cecal colonization and fecal shedding contributes to the risk of SE-positive poultry products contaminating human foods.

Cytokines are a well-established facet of the host immune response to *Salmonella* infections (8). In mouse models of *Salmonella* infection, treatment with interferon (IFN)-γ, interleukin (IL)-6, and IL-12 proteins resulted in enhanced survival and decreased bacterial colonization in the spleen and liver (7). Furthermore, these protective effects are abrogated in animals treated with the corresponding neutralizing antibodies (8).

Although less clearly understood, cytokines also appear to be involved in the avian host immune response to *Salmonella* (41,42). Intraperitoneally injected lymphokines, generated from splenic T cells of SE-immunized hens, provide protection against organ invasion of *Salmonella* in young chicks and turkey poults (12,44). Peripheral blood leukocytes isolated from adult chickens responded to SE in culture by downregulating IL-6, IL-8, and transforming growth factor (TGF)-β4 messenger-RNA (mRNA) transcription

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(16). Additionally, we observed an increase in splenic IL-18 and IFN-γ mRNA expression in day-old chicks exposed to SE (2).

Salmonella are known to reside within phagocytic vacuoles of macrophages, but can also elicit apoptosis of infected macrophages (20,27). Apoptosis occurs through several well-defined stages that represent a mechanism of programmed cell death (15). An early event in apoptosis is the breakdown in the phospholipid bilayer, where phosphatidylserine, usually located on the inner layer, becomes externalized to the outer or external layer (13). DNA damage, mediated by caspase-activated deoxyribonucleases (DNases), results in DNA fragmentation and is a classic late stage in apoptosis (36). Our laboratory has previously demonstrated associations of sequence variation in candidate genes in apoptosis pathways with organ colonization by SE. Caspase-1 was associated with cecal bacterial load (21) and inhibitor of apoptosis protein-1 was associated with bacterial load in the spleen (21,22). These associations and the established role of apoptosis in *Salmonella* infections led us to investigate what involvement apoptosis may have in SE infections in chickens.

The current study examined mRNA expression of cytokines and an antimicrobial peptide, along with changes in immune cell populations and apoptosis, in the ceca of young chicks infected with SE. Understanding cecum-specific immune response of young chicks infected with SE may provide insights to explain the persistent colonization of this organ, and open avenues to reduce the incidence of contamination in eggs and meat products.

MATERIALS AND METHODS

***Salmonella* Enteritidis.** Nalidixic acid–resistant SE phage type 13a was cultured in Luria-Bertani broth (LB) broth at 37 C as previously described (2). Inoculum concentration was estimated with the use of an optical density measurement at 600 nm. Chicks were intraesophageally inoculated with 1×10^4 colony-forming units (cfu) in 0.25 ml LB broth at 1 day posthatch. Unexposed chicks were mock inoculated with 0.25 ml LB broth in a similar manner.

Experimental animals. Two advanced intercross lines (F_8 generation) of chicks initially produced by a cross of a broiler line (meat type) to two highly inbred light-bodied lines (Leghorn and Fayoumi) were used in the present study. Approximately 90 day-old chicks (45 per cross) were assigned wing bands and housed in pens in biosafety level–2 animal rooms. Chicks to be inoculated with SE were divided equally over two rooms. Following bacterial (60 chicks) or mock (30 chicks) inoculation, all chicks were given *ad libitum* access to both food and water throughout the duration of the experiment.

Organ harvest and bacterial counts. One week postinoculation (day 7 or day 8), equal numbers of chicks were euthanized by cervical dislocation and organs were harvested. Both ceca were aseptically removed, one placed into sterile labeled snap-cap tubes, and the other into OTC medium; the ceca were then quick-frozen in liquid nitrogen. Prior to this freezing, samples of cecal contents were obtained for bacterial culture and quantification with sterile cotton swabs. Each swab was cultured overnight at 37 C in 10 ml of selenite enrichment broth. To determine SE cfu per milliliter, 10-fold serial dilutions of enrichment broth were plated and cultured overnight at 37 C on brilliant-green agar plates that contained 100 μg/ml of nalidixic acid (17). Cecal tissue samples were stored at −70 C until they were processed for RNA isolation.

RNA isolation. Total RNA was isolated from homogenized frozen cecal tissues with the use of RNAqueous (Ambion, Austin, TX) and DNase treated with DNA-free (Ambion) according to manufacturer's instructions. Spectrophotometric absorbance at 260 and 280 nm was used to determine total RNA concentration and sample purity. From these stock total RNA isolates, 50 ng/μl dilutions were prepared and stored at −20 C prior to use in gene-expression assays.

mRNA expression. Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR), with the use of QuantiTect SYBR Green RT-PCR (Qiagen, Waltham, MA), determined the mRNA expression levels of CXCLi1[K60], CXCLi2 [IL-8/CAF], IFN-γ, IL-1β, IL-6, IL-12α, IL-12β, and gallinacin [Gal]-2. Primer sequences have been previously published for all genes (2,16,24,35,42). Quantitative real-time RT-PCR reactions, run in triplicate for each sample and gene, were performed on an Opticon 2 (MJ Research Inc., Waltham, MA), as previously described (2). Data were transformed and expressed as the adjusted Ct (cycle threshold) value with the use of the following formula:

$$40 - [(\text{mean test gene Ct}) + (\text{median 28S Ct} - \text{mean 28S Ct}) \times (\text{test gene slope}/\text{28S slope})],$$

where slopes are determined with a series of 10-fold dilutions of plasmids encoding each target gene to account for PCR efficiency, and median 28S Ct represents the median Ct value of all individual samples for this housekeeping reference gene.

Slide preparation and staining. Individual cecal lobes were placed into OTC medium and quick frozen in liquid nitrogen. Samples were stored at −70 C prior to cutting. Frozen sections approximately 6 μm thick were cut, placed on new slides, and fixed in chilled acetone. Each staining step was followed by a 5-min wash in phosphate-buffered saline, pH 7.5. Endogenous peroxidase activity was quenched by an initial incubation of 0.3% hydrogen peroxide for 30 min. With the use of a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA), slides were blocked for 20 min with normal horse serum, after which excess serum was blotted. Primary antibodies [K1 (macrophages), K55 (lymphocytes), HB2 (human T cells), single-stranded DNA (ssDNA), and normal mouse immunoglobulin M (IgM), or biotinylated annexin V protein] were incubated for 60 min. The K1 and K55 monoclonal antibodies are specific for macrophages and pan-lymphocytes, respectively, although the exact molecules recognized have not been determined (3,4). Annexin V binds to phosphatidylserine with a high level of affinity (13), and the ssDNA monoclonal antibody binds ssDNA (36). Annexin V and the ssDNA antibodies are not species-specific reagents. Concentrations used were K1 (1:10 dilution), K55 (1:25 dilution), HB2 (1:100 dilution), ssDNA (1.0 μg/ml), annexin V (1.0 μg/ml), and normal mouse IgM (5.0 μg/ml) in diluted blocking serum. The HB2 (14) and normal mouse IgM antibodies were used as negative controls. Sections were then incubated with biotinylated secondary antibody (anti-mouse IgG or IgM) for 30 min, excluding those stained with annexin V. Next, slides were incubated 30 min with Vectastatin ABC reagent. Sections were incubated for 5 min in Vector NovaRed peroxidase substrate (Vector Laboratories) and rinsed with tap water. Following a counterstain with Vector Hematoxylin QS (Vector Laboratories), slides were air dried overnight and coverslips mounted with VectaMount mounting medium (Vector Laboratories). For histologic examination of heterophils, tissues were cut, placed on slides, fixed in a manner similar to that reported above, and then stained with Vector Hematoxylin QS and Accustain eosin Y solution (Sigma-Aldrich, Inc., St. Louis, MO). Primary staining reagents were obtained from the following sources: biotinylated annexin V protein (BioVision, Mountain View, CA), ssDNA IgM isotype (Alexis Corp., Lausen, Switzerland), and normal mouse IgM (Bethyl Laboratories Inc., Montgomery, TX).

Slide imaging, staining analysis, and heterophil counts. Ceca from 24 chicks were stained and analyzed, 12 per genetic cross, representing eight infected and four uninfected with SE. Tissue sections were stained in duplicate per individual chick and staining reagent. Two separate images per slide were obtained on a Zeiss Axiophot bright-field microscope (50×). Data are represented as an average of four measurements and presented as a ratio of red-stained area (positive) to blue-stained area (negative). Any unstained (white) area was subtracted from the total image area and excluded from analysis. Heterophils, observed by hematoxylin and eosin (H&E) staining with a Spencer confocal microscope (40×), appeared round or oval in shape, had bilobulated nuclei that were bluish purple in color, and had an orange cytoplasm (23,29).

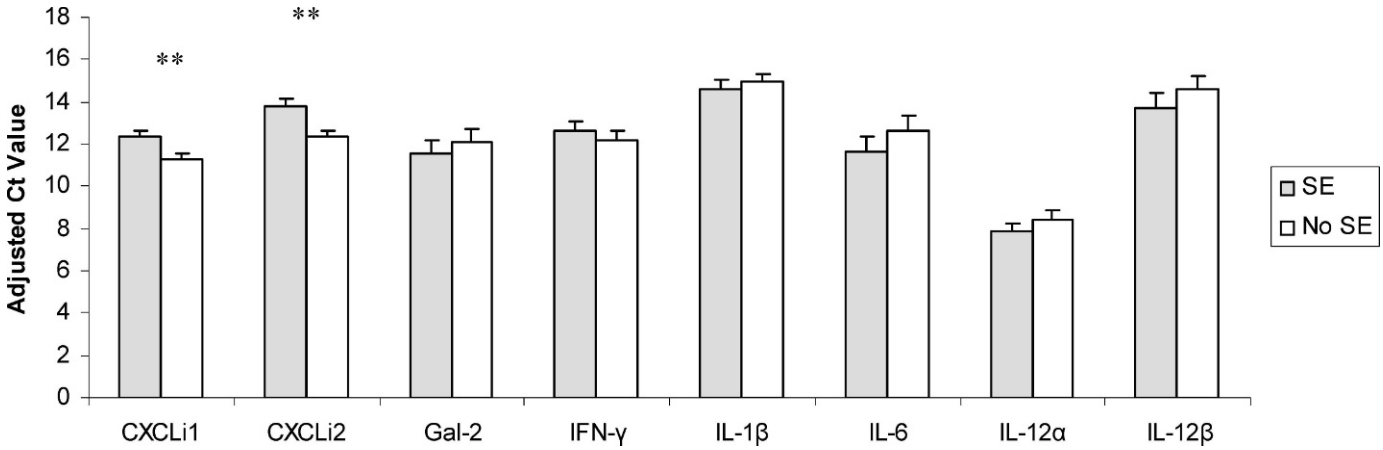

Fig. 1. Differential CXCLi1, CXCLi2, Gal-2, IFN-γ, IL-1β, IL-6, IL-12α, and IL-12β mRNA expression in the ceca of week-old chicks with or without SE infection. CXCLi1 and CXCLi2 mRNAs were significantly upregulated in SE-infected chicks compared to uninfected chicks, with *P* values less than 0.01 (**).

Statistical analysis. Gene mRNA expression and tissue staining levels were analyzed with an analysis of variance model using JMP software (33). Individual-bird mRNA data are represented as the average of triplicate measurements. Duplicate slides were prepared (on different days) for each tissue stain. Data for cecal tissue staining levels are represented as the average of four images (two per slide).

RESULTS

To confirm bacterial colonization of the cecum, we cultured cecal contents in selenite enrichment broth. All SE inoculated chicks were positive for the presence of the bacterium 1 wk after initial exposure; none of the uninfected birds cultured positive. A broad range of bacterial colonization levels (19–22 natural log) were observed for the SE-infected chicks; however, there was no significant correlation of bacterial count from the samples of cecal content and individual gene expression level for cecal tissue (data not shown).

Cytokine, chemokine, and antimicrobial peptide mRNA expression in response to SE infection was measured. From the ceca of infected and uninfected chicks, total RNA was harvested and assayed for expression of CXCLi1 [K60], CXCLi2 [IL-8/CAF], IFN-γ, IL-1β, IL-6, IL-12α, IL-12β, and Gal-2. Both CXCLi1 (also known as K60) and CXCLi2 (previously referred to as IL-8 and CAF) mRNA transcripts were upregulated in SE-infected animals compared to uninfected birds (Fig. 1). Increased inflammatory CXCL chemokine mRNA expression in the ceca of young chicks was observed 1 wk postinfection (pi) with SE. SE infection did not induce differential gene expression for IFN-γ, IL-1β, IL-6, IL-12α, IL-12β, or the antimicrobial peptide Gal-2. No significant differences in gene expression were observed between sexes or genetic lines (data not shown).

To investigate how local cell populations in the cecum are influenced by SE infection in the neonatal chick, we used immunohistochemistry to determine changes in macrophage, lymphocyte, and heterophil numbers in tissue sections. Additionally, both early and late stages of apoptosis were assayed on the tissue sections. A significant increase in total area staining positive for macrophages (K1) in the cecum was associated with SE infection, suggesting an influx or proliferation of this cell population (Figs. 2, 3). SE infection was additionally associated with a fourfold decrease in the area positive for late apoptotic cells (ssDNA) (Fig. 4). Bacterial infection did not influence the total area positive for lymphocytes (K55) or early apoptotic cells (annexin V) in the cecum (Fig. 2). Heterophil counts of SE-infected and control animals did not differ significantly, and no associations of sex or genetic line with cecal macrophage, lymphocyte, heterophil, or apoptotic cell populations were observed (data not shown).

DISCUSSION

Infection of young chicks with SE in the current study resulted in enhanced mRNA transcription of two chemokines, CXCLi1 and CXCLi2, which are similar to mammalian CXCL8 (IL-8), and have a role in inflammation (18). Although the exact function of these chicken chemokines remains to be determined, it is reasonable to postulate that these proteins recruit avian heterophils, as CXCL8 preferentially attracts mammalian neutrophils (10). However, evidence from other studies suggests that macrophages, monocytes, and lymphocytes are chemotactic targets for these CXCLi chemokines, not the avian heterophil (25,26,42). Our results provide additional support for CXCLi1 and CXCLi2 in macrophage recruitment, as we observed an increase in area of tissue staining for this cell population that coincided with an enhanced expression of these two chemokines. An increase in macrophages (K1 positive) and upregulation of CXCLi1 (K60) and CXCLi2 (IL-8) mRNA expression was also observed for intestinal intraepithelial lympho-

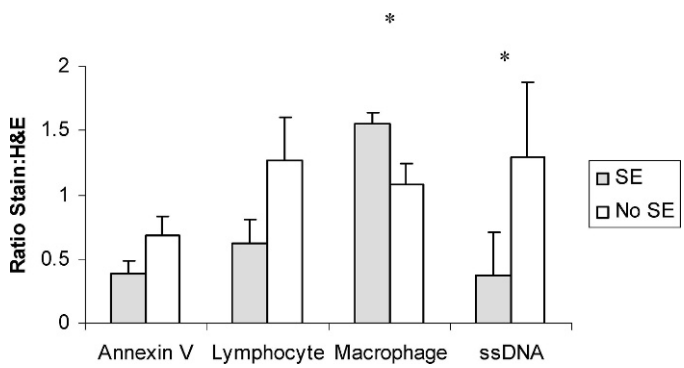

Fig. 2. Differential staining of annexin V, lymphocyte, macrophage, and ssDNA in frozen cecal tissue samples of week-old chicks with or without SE infection. Data presented as a ratio of area stained to the area counterstained with H&E. A significant increase in macrophage staining and decrease in ssDNA staining of SE-infected chicks compared to uninfected chicks is shown, with *P* values less than 0.05 (*).

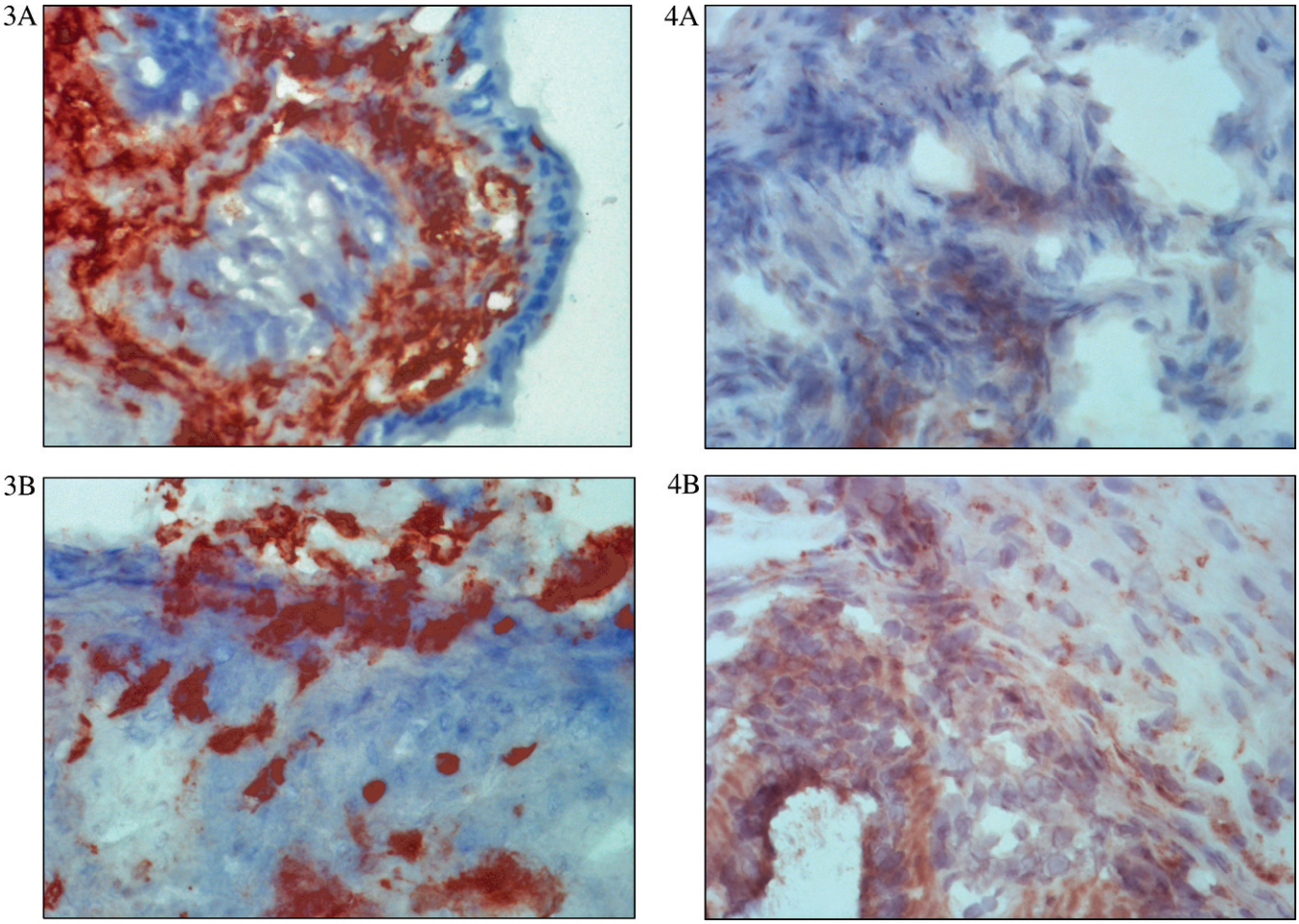

Fig. 3. Staining for the presence of macrophages in frozen cecal tissue samples of week-old chicks with (A) or without (B) SE infection. Increased staining for macrophages in infected animals (A) compared to those uninfected (B). Staining is expressed as a ratio of red-stained area (positive) to the area of blue-stained area (negative). Unstained (white) area was subtracted from the total image area and excluded from analysis. Anti-K1 immunostaining, counterstaining with Hematoxylin QS; original magnification, 50×.

Fig. 4. Staining for the presence of apoptotic cells in frozen cecal tissue samples of week-old chicks with (A) or without (B) SE infection. Increased staining for ssDNA in uninfected animals (B) compared to those infected (A). Staining is expressed as a ratio of red-stained area (positive) to the area of blue-stained area (negative). Unstained (white) area was subtracted from the total image area and excluded from analysis. Anti-ssDNA immunostaining, counterstaining with Hematoxylin QS; original magnification, 50×.

cytes 7 days pi with *Eimeria maxima* (14), indicating these responses occur broadly across different intracellular pathogen classes (bacteria and protozoan) and in different areas of the gastrointestinal tract and may represent generalized responses to pathogens in the avian gut.

SE infection did not influence IFN-γ, IL-1β, IL-6, IL-12, or Gal-2 gene expression in the ceca of young chicks (Fig. 1). These results seem to contradict published reports of the involvement of these genes in immune responses to *Salmonella* in poultry (19,26) and mice or humans (8); however, this may be because of the specific time points measured. Our study examined only one time point, 1 wk after SE infection, and we did not determine the kinetics of cytokine gene expression. Heterophils, which produce antimicrobial peptides such as Gal-2 (1,9), did not significantly increase in numbers in ceca of SE-infected chicks; therefore, a lack of Gal-2 mRNA upregulation in the tissue of these infected animals is not surprising. SE infection did not alter IL-1β and IL-6 mRNA expression, and this may reflect both the young age at infection (1 day) and the tissue harvest time (1 wk pi). Primary infection in chickens with *Salmonella* Typhimurium induced an upregulation of IL-6 mRNA expression at 14–28 days pi, but upon secondary infection IL-6 mRNA was rapidly expressed from 1 to 7 days pi (43), suggesting that IL-6 mediates a later and predominantly secondary immune response in the ceca of *Salmonella* Typhimurium–infected chickens.

Activation of macrophages from various external stimuli and cytokine production results in enhanced effector functions such as reactive oxygen intermediate production, phagocytosis, and cytotoxicity toward microbes and tumor cells (30,31). Cytokines such as IL-6, IL-12, and IFN-γ promote macrophage activation and are also produced by these cells and other cell types, such as natural killer and T cells for IFN-γ (37,38). Both IL-12 and IFN-γ also promote T helper–1 immune responses that are critical for intracellular infections with viruses and bacterial species like *Salmonella* (8,38). The current study on SE infection in the ceca of young chickens found no significant differences in gene expression for IL-6, IL-12, and IFN-γ in response to pathogen challenge (Fig. 1). Although an unexpected result, we suggest that this could reflect the activation state of macrophages in the cecum. Although we do observe an increase of macrophage cells (as measured by total area staining positive for these cells) in the cecum following SE infection (Figs. 2,

3), the actual source (influx of recruited cells or proliferation *in situ*) is not known. The activation state of these cells would greatly influence the local immune response. Activated macrophages, rather than rapidly migrating or proliferating cells, would be better equipped to control and eliminate the bacterial challenge presented by SE in the chicken cecum.

Macrophages are a target cell of *Salmonella* in which bacteria invade and successfully replicate, allowing for spread of infection (8,20,32). The ability to influence host macrophage responses would be very beneficial to the invading microbe, and *Salmonella* are well known to do this by delaying phagosome maturation in macrophages (40). Infection with SE was associated with a decreased level of late-stage apoptosis (ssDNA staining) in the current study (Figs. 2, 4). Although the exact cells that are affected by the observed reduction in late-stage apoptosis are unknown, it would be of benefit to the pathogen to have access to nonactivated and long-lived macrophages in which to infect and replicate rapidly. *Salmonella* rapidly cause macrophage apoptosis; however, it has been suggested that during a systemic infection this would be detrimental to the pathogen and delaying apoptosis of macrophages would allow for the time required by the bacteria to replicate in sufficient numbers, escape, and eventually invade other macrophages (20). This strategy is yet to be fully investigated, especially in nonmammalian species such as chickens, but we hypothesize that our novel observation of decreased late apoptosis may be a mechanism contributing to persistent cecal colonization in poultry.

Our results in the present study show that SE infection in the young chick causes upregulation of two chemokines, CXCLi1 and CXCLi2, which actively recruit cells of the monocyte/macrophage linage. Increased macrophage staining in the ceca of SE-infected chicks was observed and is likely the result of upregulation of these chemokines. The lack of differential mRNA expression of IL-6, IL-12, and IFN-γ expression suggests that these macrophages display an immature nonactivated phenotype. SE infection in these young birds decreased staining for a marker of late-stage apoptosis in the cecum. These SE-induced changes could provide an ideal environment for the bacterium to infect and replicate in the cecum without evoking a protective host immune response to clear the infection and eliminate the presence of this zoonotic pathogen.

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